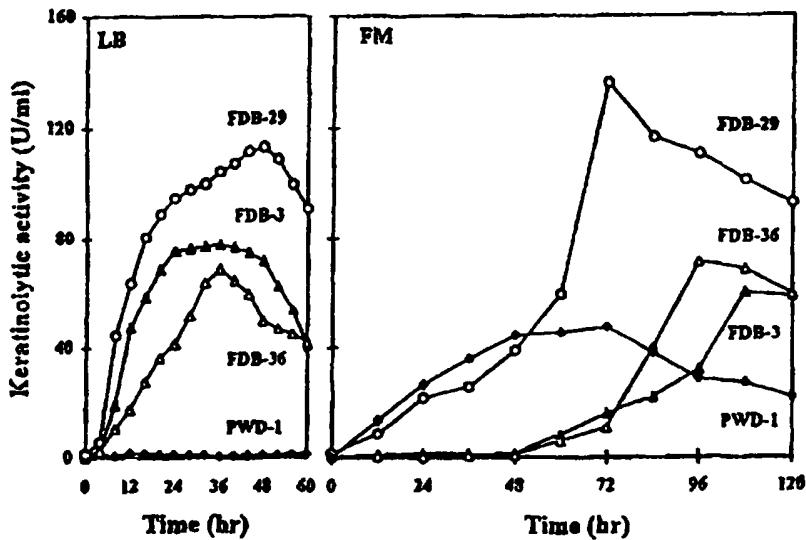




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/57, 1/21, 9/56, 15/75, 15/62		A2	(11) International Publication Number: WO 97/39130 (43) International Publication Date: 23 October 1997 (23.10.97)
(21) International Application Number: PCT/US97/06477		(74) Agents: SIBLEY, Kenneth, D. et al.; Myers, Bigel, Sibley & Sajovec, L.L.P., P.O. Box 37428, Raleigh, NC 27627 (US).	
(22) International Filing Date: 16 April 1997 (16.04.97)		(81) Designated States: AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(30) Priority Data: 08/634,440 18 April 1996 (18.04.96) US		(60) Parent Application or Grant (63) Related by Continuation US 08/634,440 (CON) Filed on 18 April 1996 (18.04.96)	
(71) Applicants (for all designated States except US): NORTH CAROLINA STATE UNIVERSITY [US/US]; 103 Holiday Hall, Campus Box 7003, Raleigh, NC 27695-7003 (US). UNIVERSITY TECHNOLOGIES INTERNATIONAL, INC. [CA/CA]; 609 14th Street, N.W., Calgary, Alberta (CA).			
(72) Inventors; and (75) Inventors/Applicants (for US only): SHIH, Jason, C., H. [US/US]; 100 Planters Wood Lane, Cary, NC 27511 (US). LIN, Xiang [CN/US]; Apartment 203, 3608 Horton Street, Raleigh, NC 27607 (US). WONG, Sui-Lam [CA/CA]; 38 Ranch Glen Drive, N.W., Calgary, Alberta T3G 1E3 (CA).			

(54) Title: METHOD FOR EXPRESSING AND SECRETING KERATINASE



(57) Abstract

The present invention provides a *Bacillus subtilis* host cell capable of expressing and secreting keratinase. The host cell contains a recombinant DNA molecule comprising vector DNA and DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme operatively associated therewith. The present invention also provides a method for producing keratinase enzyme. The method includes the steps of (a) culturing a *Bacillus subtilis* host cell containing a recombinant DNA molecule comprising vector DNA and DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme operatively associated therewith, and (b) collecting keratinase enzyme from the cell culture.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

-1-

METHOD FOR EXPRESSING AND SECRETING KERATINASE

This invention was made with Government support under grant number NRI-93-37500-9247 from the United States Department of Agriculture. The government has certain rights to this invention.

5 **Field of the Invention**

The present invention relates to cloning and expression of enzymes in and secretion by host cells, and in particular to cloning, expression, and secretion of keratinase in host cells.

10 **Background of the Invention**

Feathers are produced in large quantities by the poultry industry. These feathers provide an inexpensive source of raw material for a variety of potential uses. Among other things, there has been considerable interest in developing methods of degrading feathers so they can be used as an inexpensive source of amino acids and digestible protein in animal feed. Processes for converting feather into animal feed which have been developed to date include both steam hydrolysis processes and combined steam hydrolysis and enzymatic processes. See, e.g., Papadopoulos, M.C., *Animal Feed Science and Technology* 16:151 (1986); Papadopoulos, M.C., *Poultry Science* 64:1729 (1985); Alderibigde, A.O. et al., *J. Animal Science* 1198 (1983); Thomas and Beeson, J. *Animal Science* 45:819 (1977); Morris et al., *Poultry Science* 52:858 (1973); Moran et al., *Poultry Science* 46:456 (1967); Davis et al., *Processing of poultry by-products and their utilization in feeds, Part I, USDA Util. Res. Rep. no. 3, Washington, D.C. (1961).* Disadvantages of these procedures, such as the

-2-

degradation of heat sensitive amino acids by steam processes and the relatively low digestibility of the resulting products, have lead to continued interest in economical new feather degradation procedures which do 5 not require a harsh steam treatment.

Keratinase enzyme has been found to be an effective feather degrading enzyme useful for converting keratin into amino acids for inclusion into animal feeds. U.S. Patent Application Serial No. 10 08/250,028 filed 27 May 1994 discloses an isolated *Bacillus licheniformis* PWD-1 keratinase enzyme for such use.

It is an object of the present invention to provide new, economical methods of producing 15 keratinase.

It is a further object of the present invention to provide a host cell, and expression and secretion system for keratinase, which is capable of the hyperproduction of keratinase.

20 It is a further object of the present invention to provide recombinant DNA, host cells, and an expression and secretion system capable of hyperexpressing an enzyme encoded by a heterologous DNA.

25

Summary of the Invention

The foregoing objects are met by the present invention. As a first aspect, the present invention provides a *Bacillus subtilis* host cell capable of expressing and secreting keratinase. The host cell 30 contains a recombinant DNA molecule comprising vector DNA and DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme operatively associated therewith. *Bacillus licheniformis* PWD-1 keratinase enzyme has the sequence as set forth in SEQ ID NO:1. In the preferred 35 embodiment, the vector DNA further comprises a *kerA*

-3-

pre/pro processing and secretion region at nucleotides 215 through 529 of the keratinase gene (SEQ ID NO:1).

As a second aspect, the present invention provides a method for producing keratinase enzyme. The 5 method includes the steps of (a) culturing a *Bacillus subtilis* host cell containing a recombinant DNA molecule comprising vector DNA and DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme operatively associated therewith, and (b) collecting 10 keratinase enzyme from the cell culture.

As a third aspect, the present invention provides an expression and secretion system for keratinase enzyme. The expression and secretion system includes (a) a *Bacillus subtilis* host cell, and (b) a 15 recombinant DNA molecule comprising vector DNA, DNA encoding a *kerA* pre/pro processing and secretion region, and DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme operatively associated therewith.

As a fourth aspect, the present invention 20 provides a recombinant DNA molecule comprising vector DNA, DNA encoding a *kerA* pre/pro processing and secretion region, and a heterologous DNA encoding an enzyme. The heterologous DNA encoding the enzyme may be a heterologous DNA encoding a proteinase, in 25 particular a keratinase.

As a fifth aspect, the present invention provides a *Bacillus subtilis* host cell capable of expressing and secreting an enzyme encoded by a heterologous DNA. The host cell contains a recombinant 30 DNA molecule comprising vector DNA, DNA encoding a *kerA* pre/pro processing and secretion region, and a heterologous DNA encoding an enzyme. Preferably, the heterologous DNA is a heterologous DNA which does not encode *Bacillus licheniformis* PWD-1 keratinase enzyme.

As a sixth aspect, the present invention 35 provides a method of producing an enzyme. The method includes the steps of (a) culturing a *Bacillus subtilis*

host cell containing a recombinant DNA molecule comprising vector DNA, DNA encoding a *kerA* pre/pro processing and secretion region, and a heterologous DNA encoding an enzyme, and (b) collecting enzyme from the 5 *Bacillus subtilis* host cell culture. Preferably, the heterologous DNA is a heterologous DNA which does not encode *Bacillus licheniformis* PWD-1 keratinase enzyme.

As a seventh aspect, the present invention provides an expression and secretion system for an 10 enzyme. The system includes (a) a *Bacillus subtilis* host cell, and (b) a recombinant DNA molecule comprising vector DNA, DNA encoding a *kerA* pre/pro processing and secretion region, and a heterologous DNA encoding an enzyme. Preferably, the heterologous DNA 15 is a heterologous DNA which does not encode *Bacillus licheniformis* PWD-1 keratinase enzyme.

The foregoing and other objects and aspects of the present invention are explained in detail in the detailed description set forth below.

20

Brief Description of the Drawings

Figure 1 illustrates the construction of a plasmid, pLB3, containing the 1.45 kilobase *kerA* keratinase gene. *Km^r* denotes the kanamycin resistance gene.

25

Figure 2 illustrates the structures of plasmids, pLB3, pLB29, and pLB36 all containing the 1.45 kilobase *kerA* keratinase gene. P43 represents the ~300 base pair fragment containing the vegetative growth promoter. *Km^r* denotes the kanamycin resistance gene. Arrows indicate the orientations of genes.

30 **Figure 3** illustrates the detection of proteolytic activity by formation of hydrolysis haloes on milk-agar plates. Plate A represents cell-free culture supernatants from 72-hour feather medium.

35 Plate B represents 36-hour cell-free culture supernatants from Luria-Bertani medium. The numbers on

-5-

the plates represent culture supernatants from (1) PWD-1, (2) FDB-3, (3) FDB-29, (4) FDB-36, and (5) DB104/pUB18.

Figure 4 is a graphical illustration of the expression of *kerA* in FDB-3, FDB-29, and FDB-36 in Luria-Bertani (LB) medium and feather medium (FM). Keratinolytic activity was measured by azokeratin hydrolysis.

Figure 5 illustrates the immuno-diffusion assay of keratinase produced in culture media using rabbit anti-keratinase serum. Plate A contains cell-free culture supernatants from feather medium. *Bacillus licheniformis* PWD-1 and FDB-29 samples were taken at 72 hours, FDB-3, FDB-36, and DB104/pUB18 samples were taken at 96 hours. Plate B contains 36-hour cell-free culture supernatants from LB medium. The numbers on plates represent culture supernatants from (1) PWD-1, (2) FDB-3, (3) FDB-29, (4) FDB-36, and (5) DB104/pUB18.

Figures 6A, 6B, and 6C are graphical illustrations of the effects of kanamycin on *kerA* expression. Figure 6A represents results obtained from bacterial strain FDB-3 in Luria-Bertani (LB) medium and feather medium (FM). Figure 6B represents results obtained from bacterial strain FDB-29 in Luria-Bertani (LB) medium and feather medium (FM). Figure 6C represents results obtained from bacterial strain FDB-36 in Luria-Bertani (LB) medium and feather medium (FM).

30 Detailed Description of the Invention

Amino acid sequences disclosed herein are presented in the amino to carboxy direction, from left to right. The amino and carboxy groups are not presented in the sequence. Nucleotide sequences are 35 presented herein by single coding strand only, in the 5' to 3' direction, from left to right. Nucleotides

- and amino acids are represented herein in the manner recommended by the IUPAC-IUB Biochemical Nomenclature Commission, or (for amino acids) by three letter code, in accordance with 37 CFR §1.822 and established usage.
- 5 See, e.g., PatentIn User Manual, 99-102 (Nov. 1990) (U.S. Patent and Trademark Office, Office of the Assistance Commissioner for Patents, Washington, D.C. 20231); U.S. Patent No. 4,871,670 to Hudson et al., at Col. 3, lines 2-=43 (applicants specifically intend 10 that the disclosure of this and all other patent references cited herein be incorporated herein by reference).

A. **DNA Encoding Keratinase Enzyme**

DNA molecules which encode a keratinase 15 enzyme are those which encode a protein capable of degrading a keratin source such as feathers. This definition is intended to encompass natural allelic variations in the DNA molecules. As used herein, "natural" or "native" DNA refers to sequences isolated 20 from natural sources, as opposed to sequences created by chemical synthesis and not found in nature.

Hybridization conditions which will permit other DNA sequences which code on expression for a keratinase to hybridize to a DNA sequence as given herein are, in general, high stringency conditions. 25 For example, hybridization of such sequences may be carried out under conditions represented by a wash stringency of 0.3 M NaCl, 0.03 M sodium citrate, 0.1% SDS at 60°C or even 70°C to DNA disclosed herein in a standard *in situ* hybridization assay. See, J. Sambrook 30 et al., Molecular Cloning, A Laboratory Manual (2nd. Ed. 1989) (Cold Spring Harbor Laboratory)). In general, DNA sequences which code for a keratinase and hybridize to the DNA sequence encoding the *Bacillus licheniformis* PWD-1 keratinase disclosed herein will be 35 at least 65%, 70%, 75%, 80%, 85%, 90%, or even 95%

-7-

homologous or more with the sequence of the keratinase DNA disclosed herein.

Further, DNA sequences (or oligonucleotides) which code for the same keratinase as coded for by the foregoing sequences, but which differ in codon sequence from these due to the degeneracy of the genetic code, are also an aspect of this invention. The degeneracy of the genetic code, which allows difference nucleic acid sequences to code for the same protein or peptide, is well known in the literature. See, e.g., U.S. Patent No. 4,757,006 to Toole et al. at Col. 2, Table 1.

DNA sequences (or oligonucleotides) which code for the same keratinase as coded for by the foregoing sequences, but which differ in codon sequence from these due to site directed mutagenesis are also contemplated by this invention. Site directed mutagenesis techniques useful for improving the properties of the keratinase enzyme are well known, as described below. See, e.g., U.S. Patent No. 4,9873,192 to Kunkel.

As used herein, "kerA" refers to the 1.457 kilobase keratinase gene encoding keratinase and including the *kerA* pre/pro processing and secretion region. The nucleotide sequence for *kerA* gene is set forth in SEQ ID NO.:1. The amino acid sequence encoded by *kerA* is set forth in SEQ ID NO.:2. Also as used herein, "kerA pre/pro processing and secretion region" refers to the nucleotide sequence from nucleotide 215 to nucleotide 529 of the *kerA* gene, which comprises the pre-region (nucleotides 215-301) and the pro-region (nucleotides 302-529). The processing and secretion region of keratinase permit the cleavage and the extracellular secretion of the expressed protein. The pre-region of *kerA* encodes a signal peptide for secretion of the protein. The pro-region of *kerA* encodes a signal peptide which controls correct folding of the

-8-

peptide. The mature protein of kerA extends from nucleotide 530 to nucleotide 1351, and encodes the 274 amino acid keratinase.

B. Genetic Engineering Techniques

5 The production of cloned genes, recombinant DNA, vectors, transformed host cells, proteins and protein fragments by genetic engineering is well known. See, e.g., U.S. Patent No. 4,761,371 to Bell et al. at Col. 6 line 3 to Col. 9, line 65; U.S. Patent No. 10 4,877,729 to Clark et al. at Col. 4, line 38 to Col. 7, line 6; U.S. Patent No. 4,912,038 to Schilling at Col. 3, line 26 to Col. 14, line 12; and U.S. Patent No. 4,879,224 to Wallner at Col. 6, line 8 to Col. 8, line 59.

15 The DNA encoding keratinase may be made according to any of the known techniques. For example, the DNA may be constructed using the MUTA-GENE™ phagemid *in vitro* mutagenesis kit by BIO-RAD. The kit is based on the method described by Kunkel in U.S. 20 Patent No. 4,873,192. (See also T. Kunkel, Proc. Natl Acad. Sci. USA 82:488 (1985); T. Kunkel et al., Methods in Enzymol. 154:367 (1987)). U.S. Patent No. 4,873,192 provides a very strong selection against the non-mutagenized strand of a double-stranded DNA. When DNA 25 is synthesized in a *dut-ung*-double mutant bacterium, the nascent DNA carries a number of uracils in thymine positions as a result of the *dut* mutation, which inactivates the enzyme DUTPase and results in high intracellular levels of DUTP. The *ung* mutation 30 inactivates uracil N-glycosylase, which allows the incorporated uracil to remain in the DNA. This uracil-containing strand is then used as the template for the *in vitro* synthesis of a complementary strand primed by an oligonucleotide containing the desired mutation. 35 When the resulting double-stranded DNA is transformed into a cell with a proficient uracil N-glycosylase, the

-9-

uracil-containing strand is inactivated with high efficiency, leaving the non-uracil-containing survivor to replicate (See generally, BIO-RAD catalog number 170-3576 instruction manual).

5 The keratinase gene encompassing the DNA encoding keratinase as well as regulatory elements may be constructed by amplification of a selected, or target, nucleic acid sequence. Amplification may be carried out by any suitable means. See generally, D. 10 Kwoh and T. Kwoh, *Am. Biotechnol. Lab.* 8:14 (1990). Examples of suitable amplification techniques include, but are not limited to polymerase chain reaction, ligase chain reaction, strand displacement amplification (see generally, G. Walker et al., *Proc. 15 Natl. Acad. Sci. USA* 89:392 (1992); G. Walker et al., *Nucleic Acids Res.* 20:1691 (1992)), transcription-based amplification (see, D. Kwoh et al., *Proc. Natl. Acad. Sci. USA* 86:1173 (1989)), self-sustained sequence replication (or "3SR") (see, J. Guatelli et al., *Proc. 20 Natl. Acad. Sci. USA* 87:1874 (1990)), the Q β replicase system (see, P. Lizardi et al., *Biotechnology* 6:1197 (1988)), nucleic acid sequence-based amplification (or "NASBA") (see, R. Lewis, *Genetic Engineering News* 12 9:1 (1992)), the repair chain reaction (or "RCR") (see, 25 R. Lewis, *supra*), and boomerang DNA amplification (or "BDA") (see R. Lewis, *supra*). Polymerase chain reaction is currently preferred.

DNA amplification techniques such as the foregoing can involve the use of a probe, a pair of 30 probes, or two pairs of probes which specifically bind to DNA encoding the desired target protein.

Polymerase chain reaction (PCR) may be carried out in accordance with known techniques. See, e.g., U.S. Patents Nos. 4,683,195; 4,683,202; 35 4,800,159; and 4,965,188. In general, PCR involves, first, treating a nucleic acid sample (e.g., in the presence of a heat stable DNA polymerase) with one

-10-

oligonucleotide primer for each strand of the specific sequence to be detected under hybridizing conditions so that an extension product of each primer is synthesized which is complementary to each nucleic acid strand,
5 with the primers sufficiently complementary to each strand of the specific sequence to hybridize therewith so that the extension product synthesized from each primer, when it is separated from its complement, can serve as a template for synthesis of the extension
10 product of the other primer, and then treating the sample under denaturing conditions to separate the primer extension products from their templates if the sequence or sequences to be detected are present.
These steps are cyclically repeated until the desired
15 degree of amplification is obtained. Detection of the amplified sequence may be carried out by adding to the reaction product an oligonucleotide probe capable of hybridizing to the reaction product (e.g., an oligonucleotide probe of the present invention), the
20 probe carrying a detectable label, and then detecting the label in accordance with known techniques, or by direct visualization on a gel.

Ligase chain reaction (LCR) is also carried out in accordance with known techniques. See, e.g., R. Weiss, *Science* 254:1292 (1991). In general, the
25 reaction is carried out with two pairs of oligonucleotide probes: one pair binds to one strand of the sequence to be detected; the other pair binds to the other strand of the sequence to be detected. Each
30 pair together completely overlaps the strand to which it corresponds. The reaction is carried out by, first, denaturing (e.g., separating) the strands of the sequence to be detected, then reacting the strands with the two pairs of oligonucleotide probes in the presence
35 of a heat stable ligase so that each pair of oligonucleotide probes is ligated together, then separating the reaction product, and then cyclically

-11-

repeating the process until the sequence has been amplified to the desired degree. Detection may then be carried out in like manner as described above with respect to PCR.

5 A vector is a replicable DNA construct. Vectors are used herein either to amplify DNA encoding a proteinase or keratinase as given herein and/or to express DNA which encodes a proteinase or keratinase as given herein. An expression vector is a replicable DNA
10 construct in which a DNA sequence encoding a proteinase or keratinase is operably linked to suitable control sequences capable of effecting the expression of the proteinase or keratinase in a suitable host. The need for such control sequences will vary depending upon the
15 host selected. Generally, control sequences include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences which control the termination of transcription and
20 translation.

Amplification vectors do not require expression control domains. All that is needed is the ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to
25 facilitate recognition of transformants.

Vectors comprise plasmids, viruses (e.g., adenovirus, cytomegalovirus), phage, and integratable DNA fragments (i.e., fragments integratable into the host genome by recombination). The vector replicates
30 and functions independently of the host genome, or may, in some instances, integrate into the genome itself. Expression vectors should contain a promoter and RNA polymerase binding sites which are operably linked to the gene to be expressed and are operable in the host
35 organism.

DNA regions are operably linked or operably associated when they are functionally related to each

-12-

other. For example, a promoter is operably linked to or operably associated with a coding sequence if it controls the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation.

Transformed host cells are cells which have been transformed or transfected with vectors containing a DNA sequence as disclosed herein constructed using 10 recombinant DNA techniques. Transformed host cells ordinarily express the proteinase or keratinase, but host cells transformed for purposes of cloning or amplifying the proteinase or keratinase DNA do not need to express the proteinase or keratinase. Suitable host 15 cells can include host cells known to those skilled in the art, such as for example prokaryote host cells including *Bacillus subtilis*.

In the methods and systems of the present invention, *Bacillus subtilis* host cells are preferred. 20 *Bacillus subtilis* is capable of secreting enzymes extracellularly. (See generally, Priest, *Bacterial. Rev.* 41:711 (1977) and Doi et al., *Trends Biotechnol.* Sept. 232 (1986). This feature allows this bacterium to serve as a host cell for expression and secretion of 25 foreign proteins in the medium, which can be conveniently rendered to downstream processing and utilization. The *Bacillus subtilis* system has not been widely utilized, because either the inserted gene is poorly regulated in general, or foreign proteins are 30 likely to be hydrolyzed by high levels of proteases produced by *Bacillus subtilis*. *Bacillus subtilis* has six extracellular proteases, neutral protease A, subtilisin (or "alkaline protease"), extracellular protease, metalloprotease, bacillopeptidase F, and 35 neutral protease B. To overcome these problems, protease-deficient strains of *Bacillus subtilis* have been developed. (See generally, Doi et al., *Trends*

-13-

Biotechnol. 4:232 (1986) and Wu et al. J. Bacteriol. 173:4952 (1991)). *Bacillus subtilis* deficient in only neutral protease, DB101, has been developed. A *Bacillus subtilis* strain deficient in two extracellular 5 protease, namely neutral protease and alkaline protease, and known as DB104 has been developed. A *Bacillus subtilis* strain deficient in five proteases, known as GP263, has been developed and has eliminated much of the total extracellular protease activity. A 10 *Bacillus subtilis* strain deficient in all six extracellular proteases, WB600, has also been constructed. Currently, DB104, or *Bacillus subtilis* deficient in two extracellular proteases, is the preferred strain for the host cells employed in the present invention.

15 Vectors for use in *Bacillus subtilis* host cells have been constructed. (See generally, Steinmetz et al., Mol. Gen. Genet. 200:220 (1985), Crutz et al., J. Bacteriol. 172:1043 (1990), and Wu et al., (1991) supra.) Preferably, *Bacillus subtilis* is transformed 20 using vectors generated from pUB18 or pUB18-P43 plasmids. A promoter commonly used in these recombinant expression vectors include the strong vegetative promoter P43. The promoter is operably associated to the DNA encoding the keratinase, i.e., 25 they are positioned so as to promote transcription of keratinase messenger RNA from the DNA.

The hyperexpression of keratinase has been observed using the *Bacillus subtilis* system where the kerA pre/pro processing and secretion region is 30 inserted upstream of the DNA encoding keratinase. Hence, this is the preferred embodiment of the instant invention.

C. Production of Keratinase Enzyme

As noted above, keratinase enzyme can be made 35 by culturing a host cell as described above under conditions that permit expression of the encoded

-14-

keratinase, and collecting the expressed keratinase. The host cell may be cultured under conditions in which the cell grows, and then cultured under conditions which cause the expression of the encoded keratinase, 5 or the cells may be caused to grow and express the encoded keratinase at the same time. The keratinase may be fused to an appropriate secretory leader sequence and secreted into the culture media and collected from the media, or the keratinase may be 10 expressed intracellularly, the cells then lysed, and the keratinase collected from the cell lysate. Preferably, the enzyme is produced into the culture medium and collected therefrom. In general, any suitable techniques for culturing and expressing a 15 transgenic protein may be used, as will be appreciated by those skilled in the art.

For example, the transformed *Bacillus subtilis* host cells may be cultured in Luria-Bertoni or feather medium, into which the expressed keratinase 20 enzyme is secreted and from which the keratinase may be collected. The *Bacillus subtilis* host cells are typically cultured at temperatures ranging from 30 to 45°C. The expressed enzyme may be collected from the medium according to techniques widely known in the art. 25 For example, the enzyme can be concentrated by ultrafiltration or ammonium sulfate precipitation, and purified by various chromatographic methods, as described in Lin et al., *Applied Environmental Microbiology* 58:3271 (1992).

According to one preferred embodiment of the 30 present invention, keratinase is produced by (a) culturing a *Bacillus subtilis* host cell containing a recombinant DNA molecule comprising vector DNA and DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme 35 operatively associated therewith; and (b) collecting keratinase enzyme from said cell culture. According to one preferred embodiment, the vector DNA further

-15-

comprises DNA encoding a *kerA* processing and secretion region. More preferably, the vector DNA further comprises a promoter, such as a P43 promoter, located upstream of the DNA encoding a *kerA* processing and secretion region. According to one preferred embodiment, the promoter is positioned in the same orientation as the DNA encoding the *Bacillus licheniformis* PWD-1 keratinase enzyme.

10 D. **Recombinant DNA and System for Expression of
a Heterologous DNA**

The present invention also provides a recombinant DNA and host cell for expressing a heterologous DNA encoding an enzyme or protein. Typically the heterologous DNA encoding an enzyme 15 comprises a heterologous DNA encoding a proteinase. Preferably, the heterologous DNA encoding an enzyme comprises a heterologous DNA encoding a keratinase. Examples of suitable heterologous DNA encoding enzymes for use in the present invention include but are not 20 limited to proteases, amylase, lipase, hexose isomerase, β -glucuronidase, and phytase.

According to the present invention, the recombinant DNA comprises vector DNA, DNA encoding a *kerA* pre/pro processing and secretion region, and the 25 heterologous DNA encoding an enzyme or protein. The vector DNA typically comprises a promoter. Any suitable promoter capable of regulating the expression of the heterologous DNA in the selected host cell may be employed. Preferably, the promoter is a P43 promoter. In the preferred embodiment of the 30 recombinant DNA of the present invention, the promoter is located upstream of the DNA encoding the *kerA* pre/pro processing and secretion region and is in the same orientation as the heterologous DNA encoding the 35 enzyme or protein.

-16-

The recombinant DNA may be transfected into a host cell to provide a host cell capable of expressing the heterologous DNA. Suitable host cells include those host cells discussed hereinabove in connection 5 with the expression and secretion of keratinase. The preferred host cell is *Bacillus subtilis*, and particularly the *Bacillus subtilis* strain which is deficient in both neutral and alkaline cellular proteases. The recombinant DNA of the present 10 invention and the host cell provide a *Bacillus* system for the expression and secretion of an enzyme or protein encoded by a heterologous DNA.

E. Methods of Expressing Heterologous DNA

The present invention also provides methods 15 of expressing a heterologous DNA encoding an enzyme or protein. The methods of the present invention include (a) culturing a *Bacillus subtilis* host cell containing a recombinant DNA molecule comprising vector DNA, DNA encoding a *kerA* pre/pro processing and secretion 20 region, and a heterologous DNA encoding an enzyme or protein, and (b) collecting enzyme or protein from the *Bacillus subtilis* host cell culture or cell culture medium. The recombinant DNA and host cell of the present invention are described in further detail 25 hereinabove.

The following examples are provided to illustrate the present invention, and should not be construed as limiting thereof. In these examples, "g" means grams, " μg " means micrograms, "l" means liters, 30 "ml" means milliliters, "g/l" means grams per liter, " $\mu\text{g}/\text{ml}$ " means micrograms per milliliter, " $^{\circ}\text{C}$ " means degrees Centigrade, "Km" means kanamycin.

Bacillus licheniformis PWD-1 has the accession number ATCC 53757. *Bacillus licheniformis* PWD-1 was grown on either 1) feather medium consisting 35 of 0.5 g/l of sodium chloride, 0.1 g/l magnesium

-17-

chloride hexahydrate, 0.06 g/l calcium chloride, 0.7 g/l KH_2PO_4 , 1.4 g/l K_2HPO_4 , 1.0 g/l tryptone, and 10 g/l chopped feathers at pH 7.0; or 2) Luria-Bertani ("LB") medium at 50°C. *Bacillus subtilis* DB104 is grown
5 according to Kawamura and Doi, *J. Bacteriol.* 160:442 (1984) and is deficient in both alkaline and neutral extracellular proteases. Specifically, *B. subtilis* DB104 was grown at 37°C on LB medium. *B. subtilis* DB104 carrying plasmid pUB18 or its derivatives, Km
10 was added to the medium at a final concentration of 20 $\mu\text{g}/\text{ml}$. *Escherichia coli* INVαF' and PCR cloning vector, pCRII, were purchased from Invitrogen Corporation, San Diego, California. *E. coli* INVαF' was grown at 37°C on LB medium supplemented with 50 $\mu\text{g}/\text{ml}$ ampicillin. TBAB
15 plates containing 20 μg Km/ml were obtained from Difco Laboratories, Detroit, Michigan and used for routine transformation. A skim milk-feather powder plate (containing 5% skim milk, 0.5% feather powder, 1% agar, and 20 μg Km/ml) were used to screen colonies producing
20 keratinase. Transformed *B. subtilis* strains were grown at 37°C on LB medium or feather medium.

EXAMPLE 1
DNA Manipulations

Mini-preparation of plasmids of pUB18, pUB18-
25 P43 and their derivatives are prepared by rapid alkaline sodium dodecyl sulfate method, according to the method of Rodriguez, Recombinant DNA Techniques, Addison-Wesley Publishing Co., (1983), the disclosure of which is incorporated herein by reference in its entirety. The 1.4 kb kerA fragment is cloned into polylinker site of plasmid pCRII and stored in *E. Coli* INVαF' as described previously by Lin et al., *Applied Environmental Microbiology* 61:1469 (1995), the disclosure of which is incorporated herein by reference
30 in its entirety. After *E. coli* INVαF' cells are grown on LB medium overnight, plasmid pCRII with kerA is
35

-18-

extracted by several mini-preparations, pooled and excised for *kerA* by *XbaI* and *SpeI* digestion. The digestion mixture is applied on 1.2% agarose gel electrophoresis for separation. *kerA* band is cut out, 5 and extracted from the gel by using an Elu-Quik DNA purification kit purchased from Schleicher & Schuell, Keene, New Hampshire. The extraction is carried out following the manufacturer's instruction. All restriction enzymes are the products of Promega 10 Corporation, Madison, Wisconsin. The construction of plasmid pLB3 containing *kerA* is set forth in **Figure 1**. *Km^r* represents the kanamycin resistance gene. Arrows indicate the orientations of the genes.

EXAMPLE 2

15 Construction of Vectors

Plasmid pUB18-P43 is created by inserting a DNA fragment (~300 bp) containing vegetative promoter P43 as described in Wang, et al., *Journal of Biological Chemistry* 259:8619 (1984), adjacent to the polycloning site of pUB18. Both plasmids pUB18 and pUB18-P43 have 20 the same polycloning site available for gene insertion. When the plasmids are digested by *HindIII* (5'-AAGCTT- 3'), four-base overhangs (5'-AGCT-3') are generated on both ends. Partial fill in with nucleotides A and G 25 generated two-nucleotides overhangs (5'-AG-3') at the ends of the linearized vectors. The 1.4-kb *kerA* fragment in pCRII flanking by *XbaI* (5'-TCTAGA-3') and *SpeI* (5'-ACTAGT-3') recognition site was excised by *XbaI-SpeI* digestion. The same single-strand overhangs 30 (5'-CTAG-3') are generated at both ends. Again, partial fill in with nucleotides T and C created another two-nucleotide overhangs (5'-CT-3') at both ends of the insert. These two separate treatments produced complementary overhangs on the vectors and 35 insert as illustrated in **Figure 1**. Vector and insert in a molar ratio of 1:2 are mixed and ligated according

-19-

to the method of Sambrook, et al., Molecular Cloning, A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, the disclosure of which is incorporated herein by
5 reference.

The structures of plasmids pLB3, pLB29, and pLB36 are set forth in **Figure 2**.

EXAMPLE 3

Cloning and Screening

10 The linearized pUB18 and pUB18-P43 created by *Hind*III digestion were flanked by overhangs 5'-AGCT-3', which is not complementary with the overhangs on *kerA* fragment generated by *Xba*I-*Spe*I digestion. However, the fill-in treatments on vectors by AG and on insert
15 by CT generated complementary two-nucleotide overhangs between vectors and *kerA* fragment to facilitate the ligation. Fill in also prevented linearized vector from religation, which reduced background colonies dramatically during the transformants screening. Using
20 skim milk-feather powder plates proved to be an efficient means of selecting transformants capable of expressing *kerA*.

EXAMPLE 3

Preparation of *B. subtilis* Competent Cells

25 *B. subtilis* DB104 competent cells are prepared as described in Dubnau et al., *Journal of Molecular Biology* 56:209 (1971), the disclosure of which is incorporated herein by reference in its entirety. *B. Subtilis* cells grown overnight on TBAB
30 plates are inoculated with 2 ml of SP1 medium according to J Spizizen, *Proc. Natl. Acad. Sci. USA* 44:1072 (1958) and Dubnau et al., *Journal of Molecular Biology* 56:209 (1971). The SP1 medium is prepared with 0.2% $(\text{NH}_4)_2\text{SO}_4$, 1.4% K_2HPO_4 , 0.6% KH_2PO_4 , 0.1% sodium
35 citrate·2H₂O, 0.02% MgSO_4 , 0.02% casamino acids, 0.1%

-20-

yeast extract, 0.005% tryptophan. One ml of pre-filtrated (0.2μ membrane) 50% glucose solution per 100 ml of SP1 medium is added after the medium is autoclaved. Cells are grown at 37°C for 3.5 to 4 hours 5 with rapid shaking at 300 rpm. A 0.5 ml culture of SP1 medium is then transferred to 4.5 ml SP2 medium (SP1 medium with additional 0.5 mM CaCl₂ and 2.5 mM MgCl₂), and grown for an additional 90 minutes. Thereafter, 50 μ l of EGTA solution (100 mM EGTA, pH 7.0) is added to 10 the SP2 medium. The cells are ready for transformation after shaking for 10 minutes.

EXAMPLE 4

Transformation of *B. subtilis* DB104 and Screening for Colonies Harboring Plasmid

15 Ligated DNA in 50 μ l is added to 0.5 ml of freshly prepared *B. subtilis* DB104 competent cells. After shaking at 200 rpm at 37°C for 90 minutes, cells are plated on TBAB plates with 20 μ g Km/ml, and incubated at 37°C overnight. Colonies grown on TBAB 20 plates are transferred to skim-milk-agar plates for further selection. The colonies having clear haloes are selected for plasmid isolation and analysis.

Transformation of *B. subtilis* using ligated pUB18-kerA and pUB18-P43-kerA DNA yielded hundreds of 25 colonies on TBAB plates. Thirty six from each group are randomly selected and transferred onto skim milk-agar feather powder plates for a secondary selection. Seven colonies from pUB18-kerA transformant group and six colonies from pUB18-P43-kerA transformant group 30 produced clear halos around colonies in 10 hour incubation at 37°C, while DB104/pUB18 and DB104/pUB18-P43 cells as controls did not show any sign of protein hydrolysis even after 48 hours. Those transformants 35 cells are then grown in LB medium containing 20 μ g km/ml for 3 hours. Cells in 2 ml culture from each clone are used for plasmid isolation.

-21-

EXAMPLE 5

Analysis of Plasmid Constructs

All plasmids isolated from halo-forming colonies displayed a 1.4 kb increase in size. When the 5 plasmids were used as templates for PCR amplifications, 1.4 kb fragments were produced in the reactions priming by Primer I and Primer II. These results confirmed that the increase in size by 1.4 kb is due to the insertion of *kerA*.

10 Plasmids pLB3, pLB29, and pLB 36 represent all new vectors isolated from halo-forming colonies. In fact, pLB3 represents all plasmids isolated from pUB18-*kerA* group because all of them have the *kerA* in the same orientation. In the pUB-P43-*kerA* group, pLB29 15 and pLB36 represent two opposite orientations of *kerA*.

To determine the orientation of *kerA* in the plasmids, Primer III was combined with either Primer I or Primer II to perform PCR amplifications. When pLB3 and pLB36 as templates, and Primer I and Primer III are 20 used, PCR amplified a 1.5 kb fragment and a 1.8 kb fragment respectively. The increases in size were due to the amplification of an additional 52 bp from original pUB18 and ~350 bp from original pUB18-P43. The presence of PCR products also proved that *kerA* in 25 pLB3 and pLB36 have the same orientation, and that they have the same orientation as the kanamycin resistance gene (*Km*^r) on the vectors. PCR using pLB29 template and Primer I and Primer III did not produce any major DNA fragment. However, when Primer I is replaced by Primer 30 II, a 1.8 kb fragment is observed on the agarose gel. These results indicate that *kerA* in pLB29 is in the same orientation with P43 promoter, but opposite to *Km*^r.

EXAMPLE 6

Identification of *kerA* in Plasmids

35 The newly constructed plasmids are digested by *Xba*I, followed by 1.2% agarose gel analysis.

-22-

Plasmids with a 1.4 kb size increase are applied to PCR amplifications. Three PCR primers are synthesized: Primer I (5'-CTCCTGCCAAGCTGAAGC-3', 18 mers) (SEQ ID NO.:3) and Primer II (5'-GATCATGGAACGGATTG-3', 17 mers) 5 (SEQ ID NO.:4), which are homologous to the upstream and downstream of *kerA*, respectively and Primer III (5'-GCCGTCTGTACGTTCCATAAG-3', 20 mers) (SEQ ID NO.:5) which is derived from the upstream DNA sequence of the polycloning site on pUB18 and pUB18-P43. PCR 10 amplifications with any two of the given primers are performed as described in Lin et al., *Applied Environmental Microbiology* 61:1469 (1995)., the disclosure of which is incorporated herein by reference in its entirety; except that the newly constructed 15 plasmids are used as templates. Approximately 156 ng plasmid DNA is used as the template in each PCR reaction.

EXAMPLE 7

Expression of *kerA* in LB and Feather Media

20 Five strains, *B. subtilis* DB104/pUB18, FDB-3 (DB104/pLB3), FDB-29 (DB104/pLB29), FDB-36 (DB104/pLB36), and *B. licheniformis* PWD-1 grew rapidly in LB medium. At 36 hours, 40 µl of supernatant from each medium is loaded into small wells on milk-agar 25 plate, and incubated at 50°C overnight. Hydrolysis haloes are only observed around the wells in which supernatants from FDB-3, FDB-29, and FDB-36 are loaded. This result is confirmed by the azo-keratin hydrolysis assay, when a 0.2 ml sample of each medium is taken at 30 every 4 hours and determined for its keratinolytic activity. Again media from all three strains showed strong activities against azokeratin, and FDB-29 gives the highest activity among all the three transformants. Both PWD-1 and DB104/pUB18 media showed no proteolytic 35 activities.

-23-

All five strains are also tested in feather media. PWD-1 and FDB-29 grew rapidly and reach their highest keratinase activity in approximately 72 hours. FDB-3, and FDB-36 did not display significant 5 keratinolytic activities until the third day, reaching their highest activities at least 24 hours later than FDB-29 did. FDB-29 still demonstrated the highest activity, which was 3 to 4 fold higher than that of PWD-1 grown on feather media at 50°C. PWD-1 showed 10 positive results only in feather media. DB104, the host strain, does not produce keratinase in either LB or feather media.

In feather media all these new strains, FDB-3, FDB-29, and FDB-36 yielded more keratinolytic 15 activity when kanamycin was not added in the medium. FDB-29 produced more keratinase in LB medium without this antibiotic. FDB-3 and FDB-36 however, demonstrated higher keratinolytic activity in LB medium when kanamycin was added.

20

EXAMPLE 8

Detection of Keratinase Activity

Two methods, hydrolysis of azokeratin and milk-agar plate assay, are used to detect keratinase activity according to the methods described in Lin et 25 al., *Applied Environmental Microbiology* 58:3271 (1992), the disclosure of which is incorporated herein by reference in its entirety. SDS gel electrophoresis is conducted as described in Laemmili, et al., *Nature* 227:680 (1970), the disclosure of which is incorporated 30 herein by reference in its entirety. Purified keratinase is used to generate anti-keratinase serum in rabbits by the standard method described in Harlow et al., Antibodies, A Laboratory Manual (1988) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. 35 This anti-serum which precipitates with keratinase is used to detect the enzyme in agar gel. DNA restriction

-24-

and agarose gel electrophoresis are performed as described by Sambrook et al., Molecular Cloning, A Laboratory Manual 2nd ed. (1988).

EXAMPLE 9

5 Confirmation of Expression of kerA

Active keratinase was produced by FDB-3, FDB-29, and FDB-36 in LB and feather media. This has been confirmed by milk-agar plate (containing 4% evaporated skim-milk, 1.5% agar, and 0.02% sodium azide) assay.

10 **Figure 3** illustrates the detection of proteolytic activity by formation of hydrolysis haloes on milk-agar plates. Plate A contains cell-free culture supernatants from feather medium. *Bacillus licheniformis* PWD-1 and FDB-29 samples were taken at 72 hours, FDB-3, FDB-36, and DB104/pUB18 samples were taken at 96 hours. Plate B contains 36-hour cell-free culture supernatants from LB medium. PWD-1 was grown at 50°C and all others were grown at 37°C.

Confirmation of the production of active 20 keratinase by FDB-3, FDB-29 and FDB-36 in LB and feather media was also obtained by azokeratine hydrolysis as illustrated by **Figure 4**. The assay was carried out in 500 ml flask with 150 ml medium. Seed cultures of FDB-3, FDB-29, FDB-36 and DB104/pUB18 were 25 grown in 10 ml LB medium with 20 µg Km/ml for 4 hours, and 1 ml of each was inoculated to 150-ml flask feather and LB media. Seed culture of PWD-1 (10 ml) were grown on LB and feather media for overnight firstly, and 1 ml of each was inoculated to LB and 30 feather media, respectively. No kanamycin was added into feather medium or PWD-1 growth media. Keratinolytic activity was measured according to the methods described in Lin et al., *Appl. Environ. Microbiol.* 58:3271 (1992).

35 Confirmation of the production of active keratinase by FDB-3, FDB-29 and FDB-36 in LB and

-25-

feather media was also obtained by immuno-precipitation assay as illustrated by **Figure 5**. The rabbit anti-keratinase serum was loaded into the holes in the center of each plate. Plate A contains cell-free culture supernatants from feather medium. *Bacillus licheniformis* PWD-1 and FDB-29 samples were taken at 72 hours, FDB-3, FDB-36, and DB104/pUB18 samples were taken at 96 hours. Plate B contains 36-hour cell-free culture supernatants from LB medium.

The double immuno-diffusion results indicate that FDB-3, FDB-29, and FDB-36 produced keratinase in both LB and feather media. PWD-1 in LB media and DB104 in both feather and LB media have negative responses in this immuno-precipitation assay.

Confirmation of keratinase activity was also achieved using SDS-polyacrylamide gel electrophoresis. The 33 kDa keratinase bands appeared on SDS-polyacrylamide gel when the media of FDB-3, FDB-29, and FDB-36 are used.

20

EXAMPLE 10

Effects of Promoter Orientation

As discussed in **Example 6**, PCR amplification analysis illustrated that P43 was installed upstream of *kerA* in pLB29 and in the same orientation as *kerA*. pLB36 has the P43 promoter in the opposite orientation from *kerA* and pLB3 does not contain the P43 promoter. The results of the keratinolytic activity of FDB-29, FDB-36, and FDB-3 cells demonstrate that the P43 promoter greatly enhanced the expression of *kerA*. Rapid cell growth of FDB-29 cells, associated with keratinolytic activity increase, was observed in feather medium. In contrast, FDB-3 and FDB-36 in feather medium show a long adaptive period, and produce most of their enzymes after 4 days of culture. Although the inventors do not wish to be bound by any particular theory, it appears that FDB-3 and FDB-36

-26-

underwent an induction process, which resulted in the eventual expression of kerA.

EXAMPLE 11

Effects of Orientation of Kanamycin Resistance Gene

5 The kanamycin resistance gene (Km^r) carried by plasmid expresses in response to kanamycin in the medium, and has an influence on the expression of kerA. In the presence of kanamycin, in both LB and feather media, FDB-29 produced slightly low activities, as 10 reported in **Figure 6B**. The decrease in kerA expression may be due to the generation of antisense RNA resulting from the readthrough of the kanamycin resistance gene. For FDB-3 and FDB-36, the increase in expression of kerA may also be caused by the same readthrough of the 15 kanamycin resistance gene, since kerA and the kanamycin resistance gene in these two vectors are in the same orientation. The results for FDB-3 and FDB-36 are reported in **Figures 6A and 6C** respectively. The same increases were not found when FDB-3 and FDB-36 were 20 grown in the feather medium. It is possible that the induction of kerA expression is crucial when they are grown on feathers. No keratinolytic activity was produced by *Bacillus licheniformis* PWD-1 in LB medium.

EXAMPLE 12

25 **Secretion of Keratinase in Protease-Deficient *Bacillus subtilis***

The DB104 host cells employed in the foregoing experiments are deficient in two major extracellular proteases, neutral and alkaline 30 proteases. The results of keratinolytic activity indicate that DB104 is able to express kerA originating from *Bacillus licheniformis* strain and secrete active keratinase into the medium at a high level. Because kerA pre/pro processing and secretion region exist 35 upstream of the keratinase structure gene, premature

-27-

keratinase in the cell must have been processed to active enzyme. These results demonstrate that the *kerA* pre/pro processing and secretion region is recognized and processed in DB104 even though it is deficient in
5 two major cellular proteases.

A similar *Bacillus subtilis*, WB600, which is deficient in six cellular proteases was also tested for expression of *kerA* in pLB29. Low enzyme activity was produced in LB medium. These results suggest that for
10 effective production of foreign protein, the host cell with high levels of extracellular proteases is harmful, but a low level of proteolytic process may be necessary for activating enzymes by limited proteolysis.

The foregoing is illustrative of the present
15 invention and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

-28-

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Shih, Jason C.H.
Lin, Xiang
Wong, Sui-Lam

(ii) TITLE OF INVENTION: Method For Expressing and Secreting
Keratinase

(iii) NUMBER OF SEQUENCES: 5

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Kenneth D. Sibley
(B) STREET: P.O. Drawer 31107
(C) CITY: Raleigh
(D) STATE: North Carolina
(E) COUNTRY: USA
(F) ZIP: 27622

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0. Version #1.30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Sibley, Kenneth D.
(B) REGISTRATION NUMBER: 31.665
(C) REFERENCE/DOCKET NUMBER: 5051-304

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (919) 420-2200
(B) TELEFAX: (919) 881-3175

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1457 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

-29-

(A) NAME/KEY: CDS
 (B) LOCATION: 215..1351

(ix) FEATURE:

(A) NAME/KEY: mat_peptide
 (B) LOCATION: 530..1351

(ix) FEATURE:

(A) NAME/KEY: misc_RNA
 (B) LOCATION: 215..301
 (D) OTHER INFORMATION: /note= "pre-region of keratinase"

(ix) FEATURE:

(A) NAME/KEY: misc_RNA
 (B) LOCATION: 302..529
 (D) OTHER INFORMATION: /note= "pro-region of keratinase"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTCCTGCCAA GCTGAAGCGG TCTATTCTATA CTTTCGAAC	TAACATTTT CTAAAACAGT	60
TATTAATAAC CAAAAAAATT TAAATTGCC CTCCAAAAAA ATAGGCCTAC CATATAATT	C	120
ATTTTTTTTC TATAATAAAAT TAACAGAATA ATTGGAATAG ATTATATTAT CCTTCTATT	T	180
AAATTATTCT GAATAAAAGAG GAGGAGAGTG AGTA ATG ATG AGG AAA AAG AGT	M	232
Met Met Arg Lys Lys Ser		
-105	-100	
TTT TGG CTT GGG ATG CTG ACG GCC TTC ATG CTC GTG TTC ACG ATG GCA		280
Phe Trp Leu Gly Met Leu Thr Ala Phe Met Leu Val Phe Thr Met Ala		
-95	-90	-85
TTC AGC GAT TCC GCT TCT GCT CAA CCG GCG AAA AAT GTT GAA AAG		328
Phe Ser Asp Ser Ala Ser Ala Glu Pro Ala Lys Asn Val Glu Lys		
-80	-75	-70
GAT TAT ATT GTC GGA TTT AAG TCA GGA GTG AAA ACC GCA TCT GTC AAA		376
Asp Tyr Ile Val Gly Phe Lys Ser Gly Val Lys Thr Ala Ser Val Lys		
-65	-60	-55
AAG GAC ATC ATC AAA GAG AGC GGC GGA AAA GTG GAC AAG CAG TTT AGA		424
Lys Asp Ile Ile Lys Glu Ser Gly Lys Val Asp Lys Glu Phe Arg		
-50	-45	-40
ATC ATC AAC GCG GCA AAA GCG AAG CTA GAC AAA GAA GCG CTT AAG GAA		472
Ile Ile Asn Ala Ala Lys Ala Lys Leu Asp Lys Glu Ala Leu Lys Glu		
-35	-30	-25
GTC AAA AAT GAT CCG GAT GTC GCT TAT GTG GAA GAG GAT CAT GTG GCC		520
Val Lys Asn Asp Pro Asp Val Ala Tyr Val Glu Glu Asp His Val Ala		
-15	-10	-5
CAT GCC TTG GCG CAA ACC GTT CCT TAC GGC ATT CCT CTC ATT AAA GCG		568

-30-

His Ala Leu Ala Gln Thr Val Pro Tyr Gly Ile Pro Leu Ile Lys Ala		
1 5 10		
GAC AAA GTG CAG GCT CAA GGC TTT AAG GGA GCG AAT GTA AAA GTA GCC		616
Asp Lys Val Gln Ala Gln Gly Phe Lys Gly Ala Asn Val Lys Val Ala		
15 20 25		
GTC CTG GAT ACA GGA ATC CAA GCT TCT CAT CCG GAC TTG AAC GTA GTC		664
Val Leu Asp Thr Gly Ile Gln Ala Ser His Pro Asp Leu Asn Val Val		
30 35 40 45		
GGC GGA GCA AGC TTT GTG GCT GGC GAA GCT TAT AAC ACC GAC GGC AAC		712
Gly Gly Ala Ser Phe Val Ala Gly Glu Ala Tyr Asn Thr Asp Gly Asn		
50 55 60		
GGA CAC GGC ACA CAT GTT GCC GGT ACA GTA GCT GCG CTT GAC AAT ACA		760
Gly His Gly Thr His Val Ala Gly Thr Val Ala Ala Leu Asp Asn Thr		
65 70 75		
ACG GGT GTA TTA GGC GTT GCG CCA AGC GTA TCC TTG TAC GCG GTT AAA		808
Thr Gly Val Leu Gly Val Ala Pro Ser Val Ser Leu Tyr Ala Val Lys		
80 85 90		
GTA CTG AAT TCA AGC GGA AGC GGA TCA TAC AGC GGC ATT GTA AGC GGA		856
Val Leu Asn Ser Ser Gly Ser Gly Ser Tyr Ser Gly Ile Val Ser Gly		
95 100 105		
ATC GAG TGG GCG ACA ACA AAC GGC ATG GAT GTT ATC AAT ATG AGC CTT		904
Ile Glu Trp Ala Thr Thr Asn Gly Met Asp Val Ile Asn Met Ser Leu		
110 115 120 125		
GGG GGA GCA TCA GGC TCG ACA GCG ATG AAA CAG GCA GTC GAC AAT GCA		952
Gly Gly Ala Ser Gly Ser Thr Ala Met Lys Gln Ala Val Asp Asn Ala		
130 135 140		
TAT GCA AGA GGG GTT GTC GTT GTA GCT GCA GCA GGG AAC AGC GGA TCT		1000
Tyr Ala Arg Gly Val Val Val Ala Ala Ala Gly Asn Ser Gly Ser		
145 150 155		
TCA GGA AAC ACG AAT ACA ATT GGC TAT CCT GCG AAA TAC GAT TCT GTC		1048
Ser Gly Asn Thr Asn Thr Ile Gly Tyr Pro Ala Lys Tyr Asp Ser Val		
160 165 170		
ATC GCT GTT GGT GCG GTA GAC TCT AAC AGC AAC AGA GCT TCA TTT TCC		1096
Ile Ala Val Gly Ala Val Asp Ser Asn Ser Asn Arg Ala Ser Phe Ser		
175 180 185		
AGT GTG GGA GCA GAG CTT GAA GTC ATG GCT CCT GGC GCA GGC GTA TAC		1144
Ser Val Gly Ala Glu Leu Glu Val Met Ala Pro Gly Ala Gly Val Tyr		
190 195 200 205		
AGC ACT TAC CCA ACG AAC ACT TAT GCA ACA TTG AAC GGA ACG TCA ATG		1192
Ser Thr Tyr Pro Thr Asn Thr Tyr Ala Thr Leu Asn Gly Thr Ser Met		
210 215 220		

-31-

GTT TCT CCT CAT GTA GCG GGA GCA GCA GCT TTG ATC TTG TCA AAA CAT	1240
Val Ser Pro His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His	
225 230 235	
CCG AAC CTT TCA GCT TCA CAA GTC CGC AAC CGT CTC TCC AGC ACG GCG	1288
Pro Asn Leu Ser Ala Ser Gln Val Arg Asn Arg Leu Ser Ser Thr Ala	
240 245 250	
ACT TAT TTG GGA AGC TCC TTC TAC TAT GGG AAA GGT CTG ATC AAT GTC	1336
Thr Tyr Leu Gly Ser Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val	
255 260 265	
GAA GCT GCC GCT CAA TAACATATTG TAACAAATAG CATATAGAAA AAGCTAGTGT	1391
Glu Ala Ala Ala Gln	
270	
TTTTAGCACT AGCTTTTCT TCATTCTGAT GAAGGTTGTC CAATATTTG AATCCGTTCC	1451
ATGATC	1457

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 379 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Met Arg Lys Lys Ser Phe Trp Leu Gly Met Leu Thr Ala Phe Met	
-105 -100 -95 -90	
Leu Val Phe Thr Met Ala Phe Ser Asp Ser Ala Ser Ala Ala Gln Pro	
-85 -80 -75	
Ala Lys Asn Val Glu Lys Asp Tyr Ile Val Gly Phe Lys Ser Gly Val	
-70 -65 -60	
Lys Thr Ala Ser Val Lys Lys Asp Ile Ile Lys Glu Ser Gly Gly Lys	
-55 -50 -45	
Val Asp Lys Gln Phe Arg Ile Ile Asn Ala Ala Lys Ala Lys Leu Asp	
-40 -35 -30	
Lys Glu Ala Leu Lys Glu Val Lys Asn Asp Pro Asp Val Ala Tyr Val	
-25 -20 -15 -10	
Glu Glu Asp His Val Ala His Ala Leu Ala Gln Thr Val Pro Tyr Gly	
-5 1 5	
Ile Pro Leu Ile Lys Ala Asp Lys Val Gln Ala Gln Gly Phe Lys Gly	
10 15 20	

-32-

Ala	Asn	Val	Lys	Val	Ala	Val	Leu	Asp	Thr	Gly	Ile	Gln	Ala	Ser	His
25					30						35				
Pro	Asp	Leu	Asn	Val	Val	Gly	Gly	Ala	Ser	Phe	Val	Ala	Gly	Glu	Ala
40					45					50		55			
Tyr	Asn	Thr	Asp	Gly	Asn	Gly	His	Gly	Thr	His	Val	Ala	Gly	Thr	Val
					60					65		70			
Ala	Ala	Leu	Asp	Asn	Thr	Thr	Gly	Val	Leu	Gly	Val	Ala	Pro	Ser	Val
					75				80			85			
Ser	Leu	Tyr	Ala	Val	Lys	Val	Leu	Asn	Ser	Ser	Gly	Ser	Gly	Ser	Tyr
					90				95			100			
Ser	Gly	Ile	Val	Ser	Gly	Ile	Glu	Trp	Ala	Thr	Thr	Asn	Gly	Met	Asp
					105		110				115				
Val	Ile	Asn	Met	Ser	Leu	Gly	Gly	Ala	Ser	Gly	Ser	Thr	Ala	Met	Lys
					120		125				130			135	
Gln	Ala	Val	Asp	Asn	Ala	Tyr	Ala	Arg	Gly	Val	Val	Val	Ala	Ala	
					140				145				150		
Ala	Gly	Asn	Ser	Ser	Gly	Asn	Thr	Asn	Thr	Ile	Gly	Tyr	Pro		
					155		160				165				
Ala	Lys	Tyr	Asp	Ser	Val	Ile	Ala	Val	Gly	Ala	Val	Asp	Ser	Asn	Ser
					170			175				180			
Asn	Arg	Ala	Ser	Phe	Ser	Ser	Val	Gly	Ala	Glu	Leu	Glu	Val	Met	Ala
					185		190				195				
Pro	Gly	Ala	Gly	Val	Tyr	Ser	Thr	Tyr	Pro	Thr	Asn	Thr	Tyr	Ala	Thr
					200			205			210			215	
Leu	Asn	Gly	Thr	Ser	Met	Val	Ser	Pro	His	Val	Ala	Gly	Ala	Ala	
					220				225				230		
Leu	Ile	Leu	Ser	Lys	His	Pro	Asn	Leu	Ser	Ala	Ser	Gln	Val	Arg	Asn
					235				240				245		
Arg	Leu	Ser	Ser	Thr	Ala	Thr	Tyr	Leu	Gly	Ser	Ser	Phe	Tyr	Tyr	Gly
					250			255				260			
Lys	Gly	Leu	Ile	Asn	Val	Glu	Ala	Ala	Ala	Gln					
					265		270								

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

-33-

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTCCTGCCAA GCTGAAGC

18

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GATCATGGAA CGGATT

17

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCCGTCTGTA CGTTCTAAG

20

-34-

That Which Is Claimed Is:

1. A *Bacillus subtilis* host cell containing a recombinant DNA molecule, wherein said recombinant DNA molecule comprises vector DNA and DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme operatively associated therewith, and capable of expressing and secreting keratinase.
2. The *Bacillus subtilis* host cell according to Claim 1, wherein said vector DNA of said recombinant DNA molecule further comprises a *kerA* pre/pro processing and secretion region.
3. The *Bacillus subtilis* host cell according to Claim 1, wherein said vector DNA of said recombinant DNA molecule further comprises a promoter.
4. The *Bacillus subtilis* host cell according to Claim 3, wherein said promoter is positioned upstream from said DNA encoding said *kerA* pre/pro processing and secretion region, and is operatively associated therewith.
5. The *Bacillus subtilis* host cell according to Claim 4, wherein said promoter is in the same orientation as said DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme.
6. The *Bacillus subtilis* host cell according to Claim 3, wherein said promoter is a P43 promoter.
7. The *Bacillus subtilis* host cell according to Claim 1, wherein said *Bacillus subtilis* host cell is deficient in at least one protease.

-35-

8. The *Bacillus subtilis* host cell according to Claim 1, wherein said *Bacillus subtilis* host cell is deficient in both neutral and alkaline proteases.

5 9. A method of producing keratinase enzyme comprising:

(a) culturing a *Bacillus subtilis* host cell containing a recombinant DNA molecule comprising vector DNA and DNA encoding *Bacillus licheniformis* PWD-1
10 keratinase enzyme operatively associated therewith; and
(b) collecting keratinase enzyme from said cell culture.

10. The method according to Claim 9, wherein said vector DNA of said recombinant DNA molecule
15 further comprises a *kerA* pre/pro processing and secretion region.

11. The method according to Claim 9, wherein said vector DNA of said recombinant DNA molecule further comprises a promoter.

20 12. The method according to Claim 11, wherein said promoter is positioned upstream from a *kerA* pre/pro processing and secretion region and is operatively associated therewith.

13. The method according to Claim 11,
25 wherein said promoter is in the same orientation as said DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme.

14. The method according to Claim 11, wherein said promoter is a P43 promoter.

-36-

15. The method according to Claim 9, wherein
said step of collecting keratinase enzyme comprises
separating said enzyme from said cell culture by a
method selected from the group consisting of
5 ultrafiltration and ammonium sulfate precipitation.

16. An expression and secretion system for
keratinase enzyme comprising:

(a) a *Bacillus subtilis* host cell, and
(b) a recombinant DNA molecule comprising
10 vector DNA, DNA encoding a *ker A* pre/pro processing and
secretion region, and DNA encoding *Bacillus*
licheniformis PWD-1 keratinase enzyme operatively
associated therewith.

17. The expression and secretion system
15 according to Claim 16, wherein said vector DNA of said
recombinant DNA molecule further comprises a promoter.

18. The expression and secretion system
according to Claim 17, wherein said promoter is located
upstream from said *kerA* pre/pro processing and
20 secretion region and operatively associated therewith.

19. The expression and secretion system
according to Claim 17, wherein said promoter is in the
same orientation as said DNA encoding *Bacillus*
licheniformis PWD-1 keratinase enzyme.

25 20. The expression and secretion system
according to Claim 17, wherein said promoter is a P43
promoter.

21. The expression and secretion system
according to Claim 16, wherein said *Bacillus subtilis*
30 host cell is deficient in at least one protease.

-37-

22. The expression and secretion system according to Claim 16, wherein said *Bacillus subtilis* host cell is deficient in both neutral and alkaline proteases.

5 23. A recombinant DNA molecule comprising vector DNA, DNA encoding a *kerA* pre/pro processing and secretion region, and a heterologous DNA encoding an enzyme, wherein said heterologous DNA encoding said enzyme is a heterologous DNA which does not encode
10 *Bacillus licheniformis* PWD-1 keratinase enzyme.

24. The recombinant DNA molecule according to Claim 23, wherein said heterologous DNA encoding an enzyme comprises a heterologous DNA encoding a proteinase.

15 25. The recombinant DNA molecule according to Claim 23, wherein said heterologous DNA encoding an enzyme comprises a heterologous DNA encoding a keratinase.

20 26. The recombinant DNA molecule according to Claim 23, wherein said vector DNA further comprises a promoter.

25 27. The recombinant DNA molecule according to Claim 26, wherein said promoter is positioned upstream from said DNA encoding said *kerA* processing and secretion region, and is operatively associated therewith.

30 28. The recombinant DNA molecule according to Claim 26, wherein said promoter is in the same orientation as said heterologous DNA encoding said enzyme.

-38-

29. The recombinant DNA molecule according to Claim 26, wherein said promoter is a P43 promoter.

30. A *Bacillus subtilis* host cell containing a recombinant DNA molecule, wherein said recombinant 5 DNA molecule comprises vector DNA, DNA encoding a *kerA* pre/pro processing and secretion region, and a heterologous DNA encoding an enzyme; wherein said heterologous DNA encoding said enzyme is a heterologous DNA which does not encode *Bacillus licheniformis* PWD-1 10 keratinase enzyme, and wherein said host cell is capable of expressing and secreting an enzyme encoded by said heterologous DNA.

31. The *Bacillus subtilis* host cell according to Claim 30, wherein said heterologous DNA 15 encoding an enzyme comprises a heterologous DNA encoding a proteinase.

32. The *Bacillus subtilis* host cell according to Claim 30, wherein said heterologous DNA encoding an enzyme comprises a heterologous DNA 20 encoding a keratinase.

33. The *Bacillus subtilis* host cell according to Claim 30, wherein said vector DNA of said recombinant DNA molecule further comprises a promoter.

34. The *Bacillus subtilis* host cell 25 according to Claim 33, wherein said promoter is positioned upstream from said DNA encoding said *kerA* processing and secretion region, and is operatively associated therewith.

-39-

35. The *Bacillus subtilis* host cell according to Claim 33, wherein said promoter is in the same orientation as said heterologous DNA encoding said enzyme.

5 36. The *Bacillus subtilis* host cell according to Claim 33, wherein said promoter is a P43 promoter.

37. The *Bacillus subtilis* host cell according to Claim 30, wherein said *Bacillus subtilis* 10 host cell is deficient in at least one protease.

38. The *Bacillus subtilis* host cell according to Claim 30, wherein said *Bacillus subtilis* host cell is deficient in both neutral and alkaline proteases.

15 39. A method of producing an enzyme comprising the steps of:

(a) culturing a *Bacillus subtilis* host cell containing a recombinant DNA molecule comprising vector DNA, DNA encoding a *kerA* pre/pro processing and 20 secretion region, and a heterologous DNA encoding an enzyme, wherein said heterologous DNA encoding said enzyme is a heterologous DNA which does not encode *Bacillus licheniformis* PWD-1 keratinase enzyme; and
(b) collecting enzyme from said *Bacillus* 25 *subtilis* host cell culture.

40. The method according to Claim 39, wherein said heterologous DNA encoding said enzyme comprises a heterologous DNA encoding a proteinase.

41. The method according to Claim 39, 30 wherein said heterologous DNA encoding said enzyme comprises a heterologous DNA encoding a keratinase.

-40-

42. The method according to Claim 39,
wherein said vector DNA further comprises a promoter.

43. The method according to Claim 42,
wherein said promoter is positioned upstream from said
5 DNA encoding said kerA processing and secretion region,
and is operatively associated therewith.

44. The method according to Claim 42,
wherein said promoter is in the same orientation as
said heterologous DNA encoding said enzyme.

10 45. The method according to Claim 42,
wherein said promoter is a P43 promoter.

46. The method according to Claim 39,
wherein said step of collecting said enzyme comprises
separating said enzyme from said cell culture by a
15 method selected from the group consisting of
ultrafiltration and ammonium sulfate precipitation..

47. An expression and secretion system for
an enzyme comprising:

20 (a) a *Bacillus subtilis* host cell, and
(b) a recombinant DNA molecule comprising
vector DNA, DNA encoding a kerA pre/pro processing and
secretion region, and a heterologous DNA encoding an
enzyme, wherein said heterologous DNA encoding said
enzyme is a heterologous DNA which does not encode
25 *Bacillus licheniformis* PWD-1 keratinase enzyme.

48. The expression and secretion system
according to Claim 47, wherein said heterologous DNA
encoding said enzyme comprises a heterologous DNA
encoding a proteinase.

-41-

49. The expression and secretion system according to Claim 47, wherein said heterologous DNA encoding said enzyme comprises a heterologous DNA encoding a keratinase.

5 50. The expression and secretion system according to Claim 47, wherein said vector DNA of said recombinant DNA molecule further comprises a promoter.

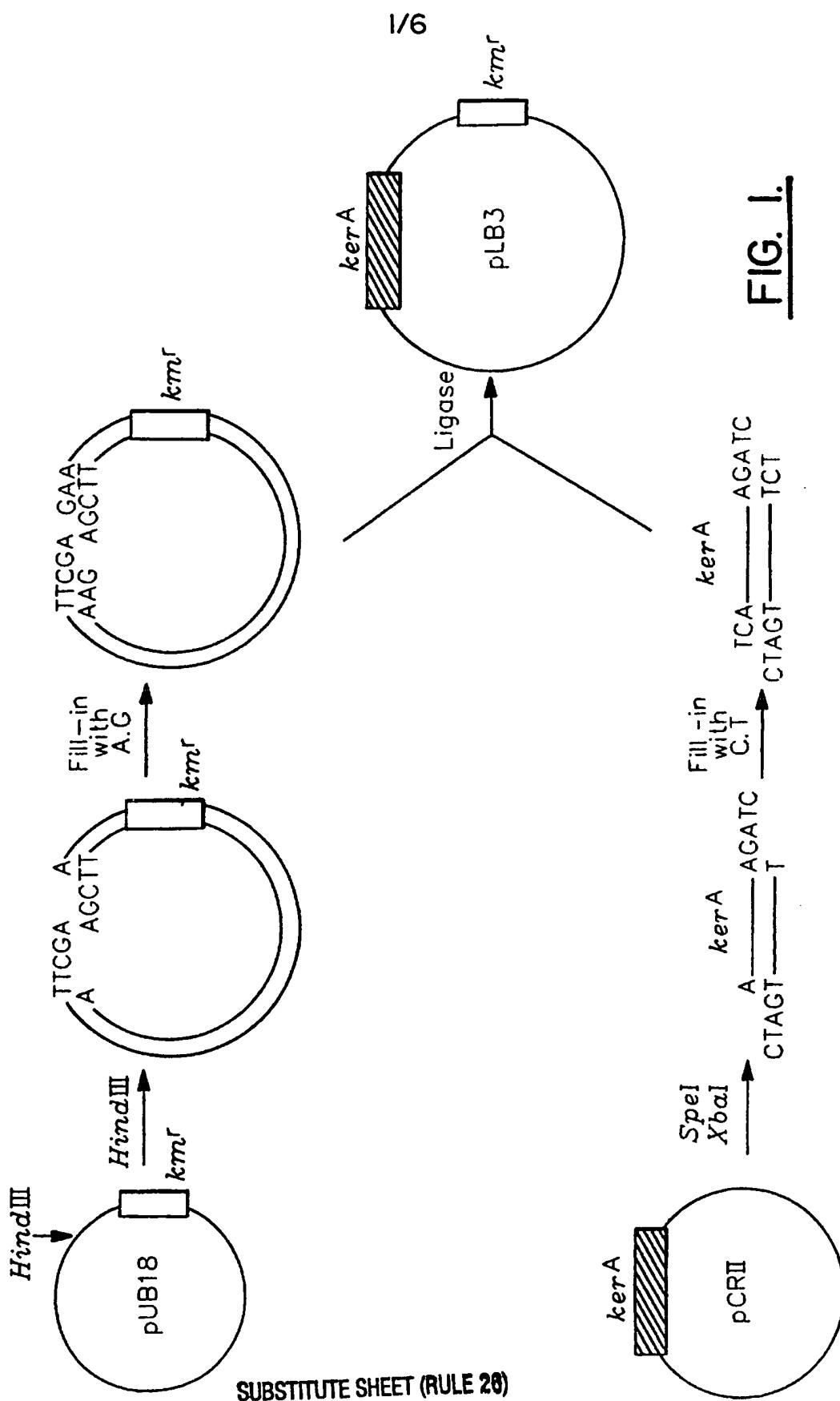
10 51. The expression and secretion system according to Claim 50, wherein said promoter is located upstream from said *kerA* pre/pro processing and secretion region, and is operatively associated therewith.

15 52. The expression and secretion system according to Claim 50, wherein said promoter is in the same orientation as said heterologous DNA encoding said enzyme.

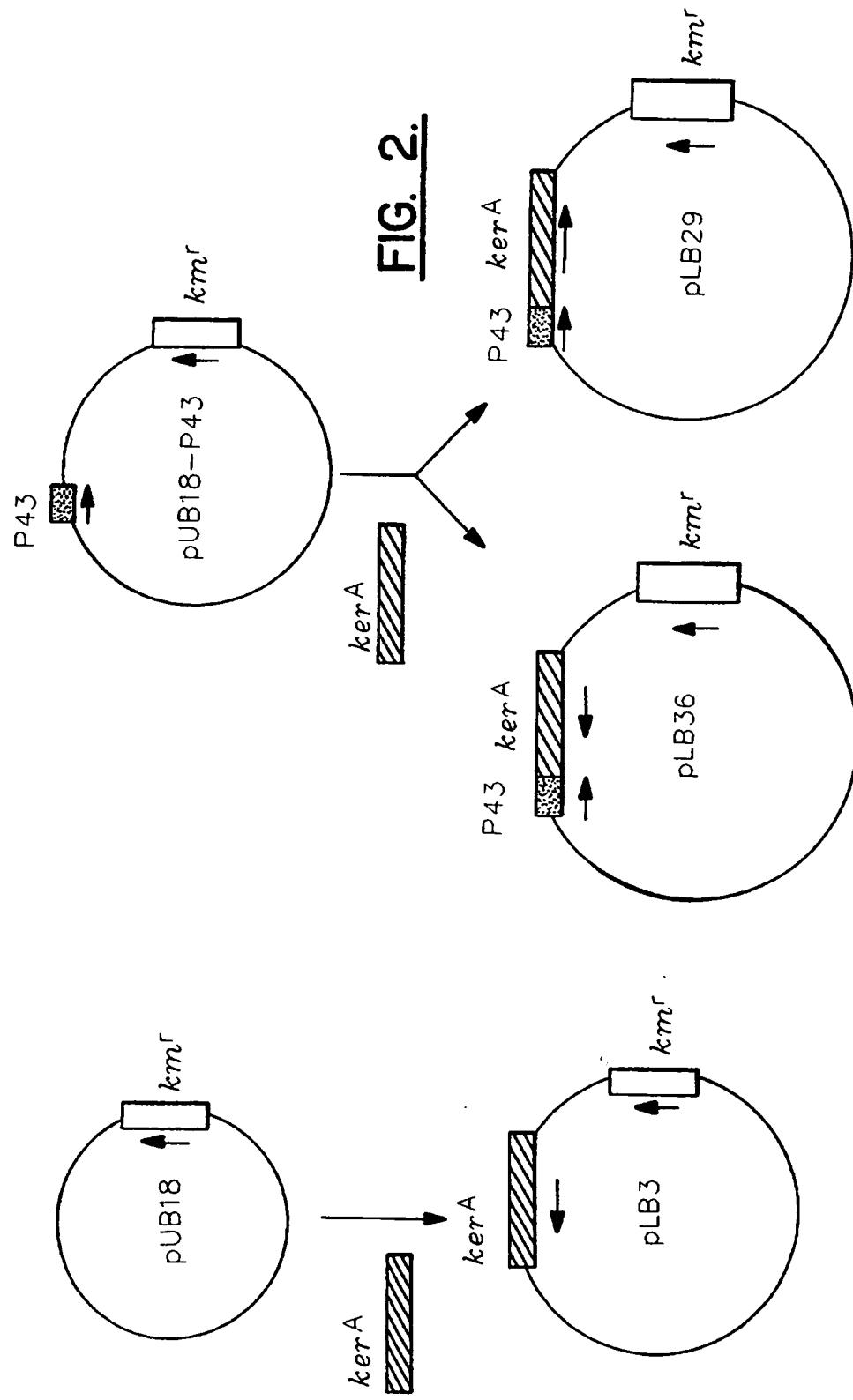
53. The expression and secretion system according to Claim 50, wherein said promoter is a P43 promoter.

20 54. The expression and secretion system according to Claim 47, wherein said *Bacillus subtilis* host cell is deficient in at least one protease.

25 55. The expression and secretion system according to Claim 47, wherein said *Bacillus subtilis* host cell is deficient in both neutral and alkaline proteases.



2/6

FIG. 2.

SUBSTITUTE SHEET (RULE 26)

3/6

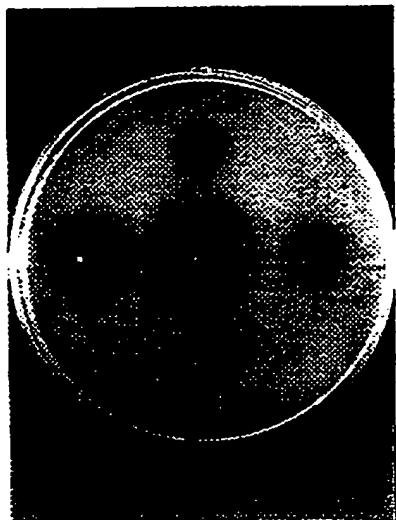


FIG.3A

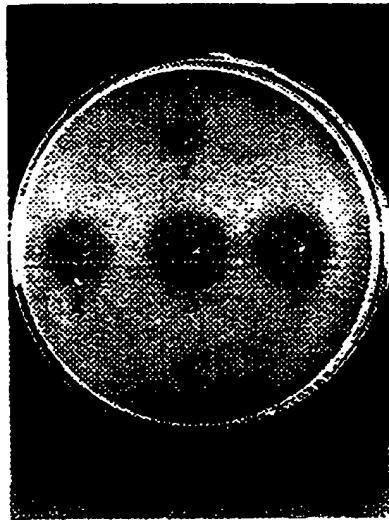


FIG.3B

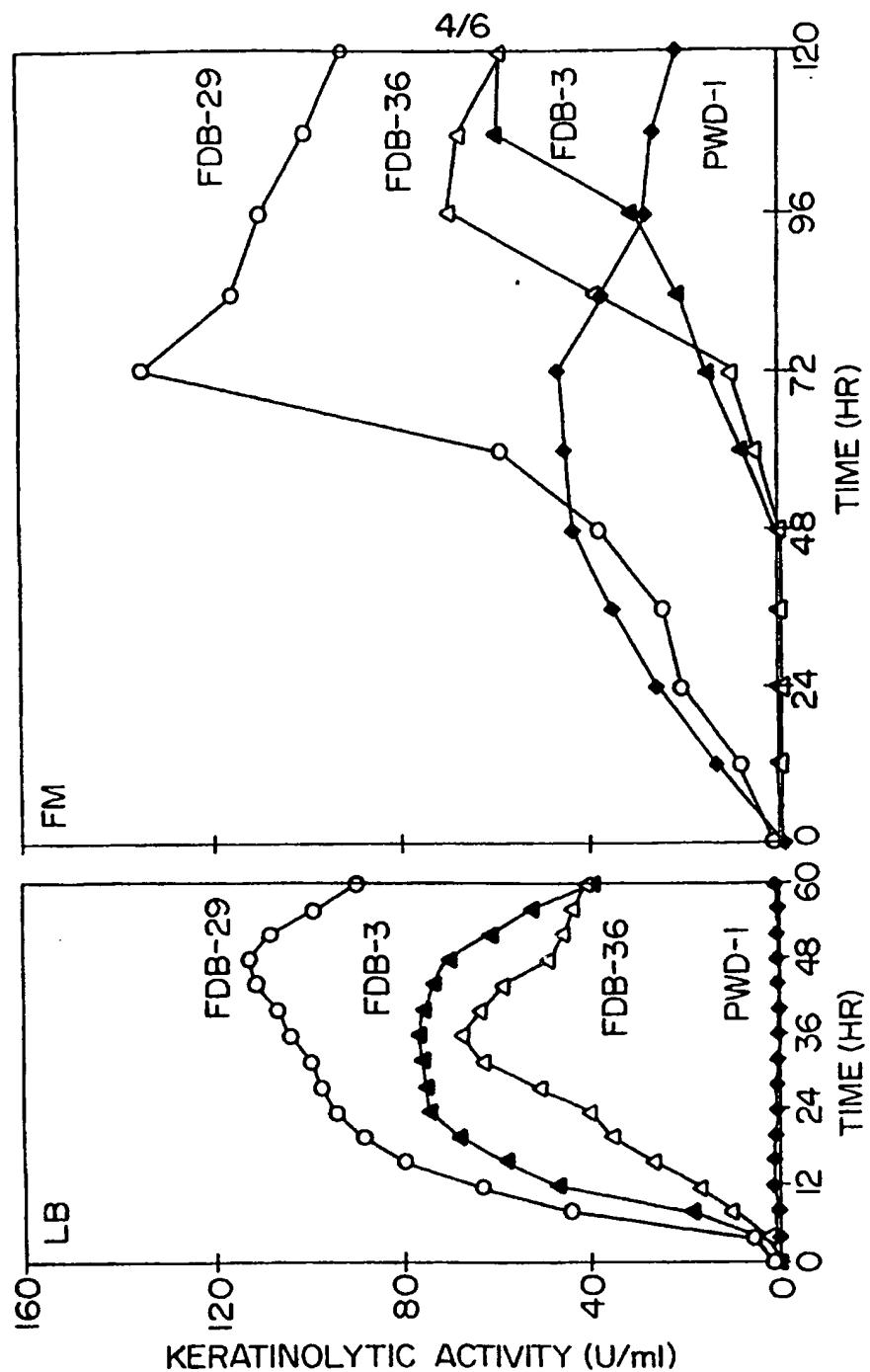


FIG. 4.

5 / 6

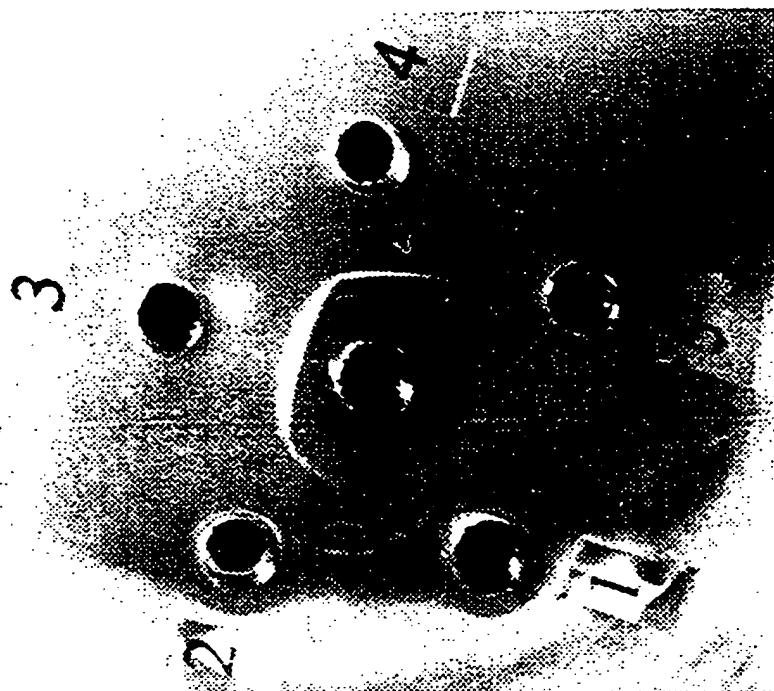


FIG. 5B.

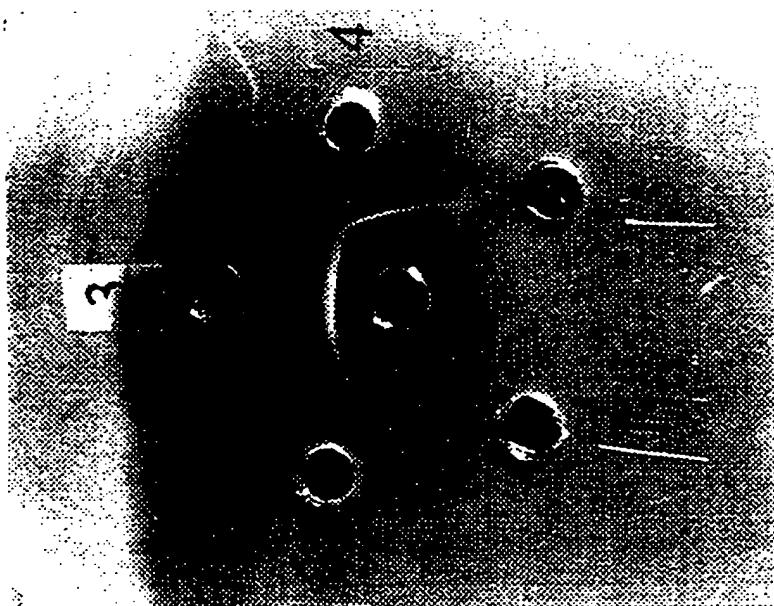


FIG. 5A.

6/6

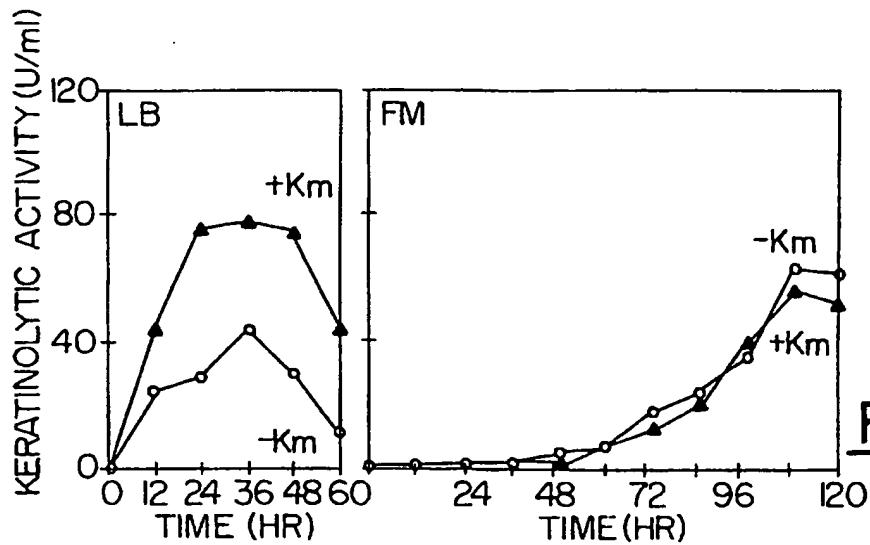


FIG. 6A.

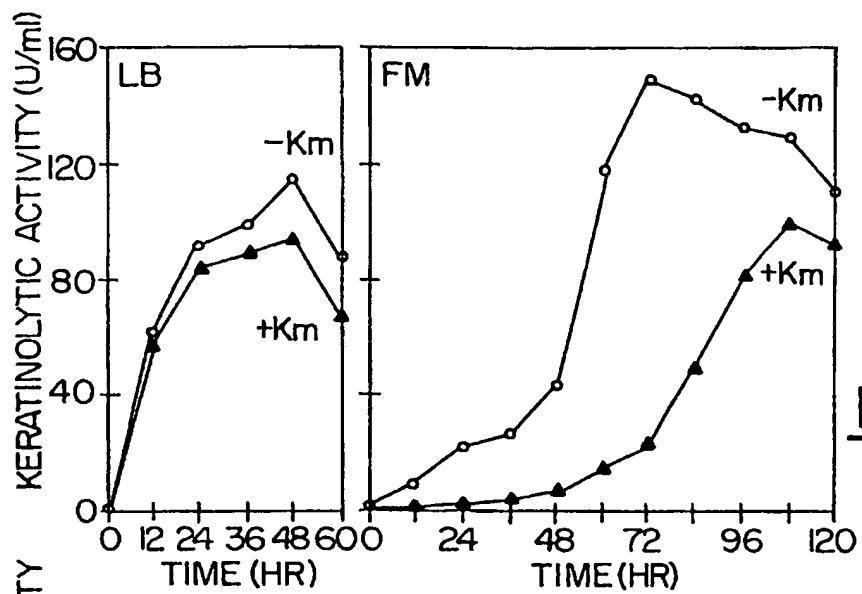


FIG. 6B.

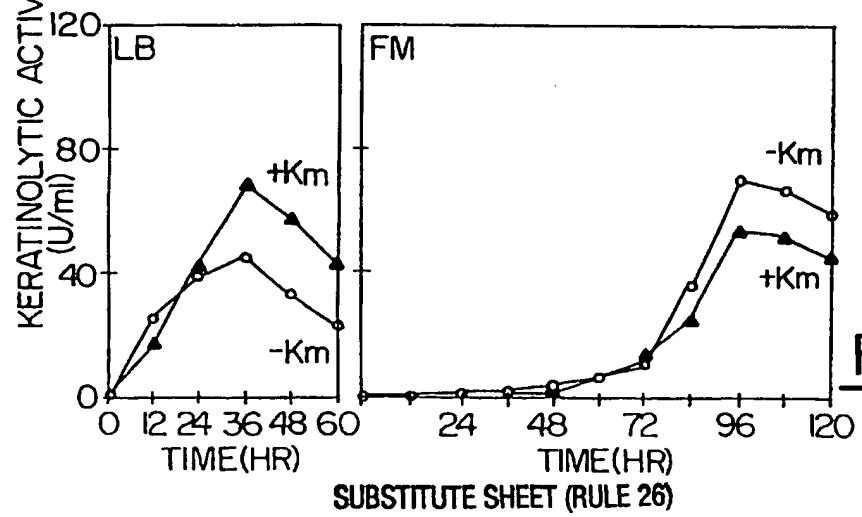


FIG. 6C.